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(71) Applicant: **Ajinomoto Co., Inc.**
No. 15-1, Kyobashi 1-chome
Chuo-ku
Tokyo (JP)

(72) Inventor: **Shimamura, Toshiro, c/o Central
Research Lab.**
Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken
(JP)

Inventor: **Nakazawa, Harumi, c/o Central
Research Lab.**
Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken
(JP)

Inventor: **Hamuro, Junji, c/o Central Research
Lab.**
Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken
(JP)

(74) Representative: **Strehl Schübel-Hopf Groening
& Partner**
Maximilianstrasse 54
D-80538 München (DE)

(54) **Polypeptide capable of inhibiting the binding between human IL-6 and its receptor.**

(57) A peptide which inhibits the binding of human IL-6 to human IL-6 receptor, a human IL-6 suppressant, anti-inflammatory agent, immunosuppressant or treatment for autoimmune diseases containing the peptide, a gene which codes for the peptide, a plasmid carrying the gene, a transformant carrying the plasmid, and a method for the production of the above peptide by culturing the above transformant.

The peptide is useful as a treatment for diseases thought to be induced or aggravated by IL-6.

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TECHNICAL FIELD

The present invention relates to a peptide which inhibits the binding of human IL-6 (interleukin-6) to human IL-6 receptor, to a polypeptide consisting essentially of the V-regions of the H-variable region of the H-chain and the L-chain of an anti-IL-6 antibody, to a human IL-6 suppressant, an anti-inflammatory agent, an immunosuppressant or an anti-autoimmune disease drug containing the above peptide, to a gene which codes for the above peptide, to a plasmid carrying the above gene, to a transformant carrying the above plasmid, and to a method for the production of the above peptide by culturing the above transformant.

DESCRIPTION OF THE PRIOR ART

Human IL-6 is a substance whose gene was isolated in 1986 (Nature, Vol.324, p.73, 1986), and it is produced by a number of different cells, including monocytes, T cells, B cells, vascular endothelial cells, fibroblasts, and the like, and is known to have a variety of physiological effects, such as the induction of the differentiation of B-cells into antibody-producing cells, the induction of acute phase protein synthesis by liver cells, the induction of the differentiation of cerebral nerve cells, the induction of the proliferation and differentiation of hematopoietic cells, etc. (Jikken Igaku, Vol.7, No.1, 1989).

Also, attention is being given to the participation of human IL-6 in various diseases, and many reports have been published in regard thereto.

First, reports regarding its participation in autoimmune diseases, include a report that the human IL-6 concentration in the synovial fluid of chronic arthritic rheumatism patients is an extremely high value in comparison with that of osteoarthritis patients (European Journal of Immunology, Vol.18, p.1797, 1988), that autoimmune disease symptoms were improved by removing the primary tumors of cardiac myxoma patients which constitutively produce IL-6 (Proceedings of National Academy of Science, U.S.A., Vol.84, p.228, 1987), and that in the case of Castleman's disease which is characterized by high γ -globulinemia, a high concentration of human IL-6 was detected in the supernatant of affected lymph node tissue cultures (Jikken Igaku, Vol.7, p.50, 1989), etc., and of the so-called autoimmune diseases of which auto-antibody production is thought to be a cause or aggravator, those with which IL-6 is strongly connected are thought to be the most dominant.

Furthermore, in the area of cancer, tumor cells taken from multiple myeloma patients constitutively produce IL-6, and their growth is further promoted by adding IL-6 thereto, which has led to a report that IL-6 is an autocrine growth factor for multiple myeloma (Nature, Vol.332, p.83, 1988), and to the suspicion that IL-6 is a growth factor for other blood tumors as well, including Lennert's T lymphoma, B-type chronic lymphocytic leukemia, etc. In addition, there is indication that the metastasis of different kinds of cancer cells to the liver in mouse models is correlated with the IL-6-producing capability of those cancer cell strains (Japanese Journal of Cancer Research, Vol.82, p.1299, 1991), and further indication of a strong correlation between IL-6 and cancer cachexia from the fact that high levels of IL-6 were shown to be present in the blood of tumor-bearing mice (Journal of Immunology, Vol.143, p.162, 1989), and that body weight loss occurred in nude mice which have been inoculated with CHO cells (Chinese hamster ovary cells) carrying the mouse IL-6 gene (Endocrinology, Vol.128, p.2657, 1991).

In the area of infections, there have been many reports regarding the connection between IL-6 and bacterial infection or viral infection, such as that the IL-6 concentration in the blood of patients with septic shock was extremely high, and patients with higher levels of IL-6 in the serum showed a lower rate of survival (Journal of Experimental Medicine, Vol.169, p.333, 1989; Blood, Vol.74, p.1704, 1989), that the mononuclear cells of fetuses suffering from intraamniotic infection produced large amounts of IL-6 (Clinical Immunological Immunopathology, Vol.55, p.305, 1990), that IL-6 was present at high levels in the cerebrospinal fluid of patients infected with the human immunodeficiency virus (HIV) (Journal of Neuroimmunology, Vol.23, p.109, 1989), and that cells derived from Kaposi's sarcoma frequently seen in AIDS patients constitutively produced IL-6, or proliferated in an IL-6-dependent manner (Proceedings of National Academy of Science, U.S.A., Vol.37, p.4063, 1990).

Furthermore, in the case of inflammatory diseases, the possibility has been suggested that IL-6 acts as an autocrine factor in mesangial proliferative glomerulonephritis, based on the fact that IL-6 was produced by mesangial cells derived from mesangial proliferative glomerulonephritis patients (Journal of Immunology, Vol.143, p.3949, 1989), and that the proliferation of mesangial cells was observed in IL-6 transgenic mice which constitutively express IL-6 in a B cell-specific manner (Proceedings of National Academy of Science, U.S.A., Vol.86, p.7547, 1989). Also, high levels of IL-6 in the serum and IL-6 production in epidermal tissue have been reported in patients with psoriasis vulgaris patients, a disease involving the proliferation of keratinocytes (Proceedings of National Academy of Science, U.S.A., Vol.86, p.6367, 1989). In addition, IL-6

is also said to contribute to the various symptoms of inflammation, such as fever, chills, malaise, etc. (American Journal of Physiology, Vol.258, p.798, 1990).

In addition to the diseases mentioned above, IL-6 is also suspected of playing a role in various other diseases, including Alzheimer's disease, amyloidosis, I-type diabetes, hyperlipidemia, osteoporosis, polycythemia vera, thrombocythemia, myocardial infarction, and the like.

Thus, human IL-6 is under much suspicion of being a cause of numerous diseases, particularly inflammatory and diseases involving the proliferation of lymphocytes, or there is a high possibility of its being involved in their aggravation, and therefore it is thought that if the functions of human IL-6 can be suppressed, then a way may be provided for the treatment of those diseases. In fact, regarding some of the above mentioned diseases, the suppression of IL-6 in mouse model experiments has led to improvement in the diseases. For example, it has been reported that, by the administration of anti-mouse IL-6 antibody or anti-mouse IL-6 receptor antibody, which have an effect of suppressing the function of mouse IL-6, to mice inoculated with myeloma cells, it is possible to both suppress the growth of myeloma cells and raise the survival rate of the mice (Journal of Experimental Medicine, Vol.172, p.997, 1990); that the metastasis of tumor cells to the liver may be suppressed by the administration of anti-mouse IL-6 antibody (Japanese Journal of Cancer Research, Vol.82, p.1299, 1991); that even mice infected with a lethal dose of *E. coli* survive when they are preadministered anti-mouse IL-6 antibody which possesses activity to suppress the function of mouse IL-6 (Journal of Immunology, Vol. 145, p.4185, 1990), that when anti-mouse IL-6 antibody which possesses mouse IL-6-suppressing activity is administered to mouse models which develop cachexia when inoculated with tumor cells, an improvement is observed not only in the weight loss, but also in the symptoms of the cachexia (Journal of Clinical Investigation, Vol.89, p.1681, 1992), etc. These facts directly indicate that IL-6 is connected with the above mentioned diseases, while they also indicate that if the function of human IL-6 can be suppressed, then a way may be provided for their treatment.

Methods which suppress the function of human IL-6 include a variety of methods such as the inhibition of IL-6 production by IL-6-producing cells, inhibition of the binding of the IL-6 produced to receptors on the IL-6 response cells, and inhibition of IL-6 signals to IL-6 response cells; however, the methods of inhibiting the binding of human IL-6 to its receptors on the human IL-6 response cells are thought to be capable of selectively suppressing only the function of human IL-6 and thus, in consideration of their clinical application, to have the least number of side effects.

Nevertheless, no medicines which specifically inhibit the binding of human IL-6 to human IL-6 receptors have been hitherto known other than mouse monoclonal anti-IL-6 antibody and mouse anti-IL-6 receptor antibody. However, these antibodies, which are the only ones known, are foreign proteins prepared from mouse hybridomas, and their constituent components are mouse proteins. Therefore, when they are administered to humans, an immune reaction is raised against the mouse protein, causing serious side effects such as anaphylactic shock, serum sickness, etc., and their effects are reduced due to the production of neutralizing antibodies against the mouse protein. Thus, the clinical application of mouse-derived antibodies for patients has been confronted with difficulty. Therefore, at present no substances are known for the specific inhibition of binding of human IL-6 to human IL-6 receptors which may be provided for clinical use, and thus it has been desired to develop clinically applicable medicines which inhibit the binding of human IL-6 to human IL-6 receptors, as treatments for the above mentioned numerous diseases.

PROBLEM TO BE SOLVED BY THE INVENTION

The purpose of the present invention is to provide a peptide which inhibits the binding of human IL-6 to human IL-6 receptor, a human IL-6 suppressant, an anti-inflammatory agent, immunosuppressant or an agent for autoimmune disease treatment containing the above peptide, a gene which codes for the above peptide, a plasmid carrying the above gene, a transformant carrying the above plasmid, and a method for the production of the above peptide by culturing the above transformant.

The present peptide may be used as a treatment for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, a treatment for bacterial infections, a treatment for septic shock due to bacterial infections, a treatment for viral infections, a treatment for cancers such as multiple myeloma, a cancer metastasis suppressant, a medicine for the amelioration of cancer cachexia, and a treatment for inflammatory diseases such as mesangial proliferative glomerulonephritis.

MEANS FOR SOLVING THE PROBLEMS

We, the inventors of the present invention, as a result of much diligent research carried out in order to overcome the above mentioned problems, have discovered, by the method described below, a peptide

which inhibits the binding of human IL-6 to human IL-6 receptor, thus suppressing the function of IL-6, and thus the present invention has been completed.

In other words, the present invention relates to a peptide which inhibits binding of IL-6 to IL-6 receptor, and particularly to a polypeptide selected from the group consisting of:

(a) a polypeptide having the following amino acid sequence:

	Met	Asp	Ile	Leu	Leu	Thr	Gln	Ser	Pro	Lys	Phe	Leu	Leu	Val	Ser	Ala	
	1				5					10					15		
10	Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Ser	Val	Ser	Thr	
				20					25					30			
	Asp	Val	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys	Leu	Leu	
			35					40					45				
	Ile	Tyr	Tyr	Ala	Ser	Asn	Arg	Tyr	Thr	Gly	Val	Pro	Asp	Arg	Phe	Thr	
15		50					55					60					
	Gly	Ser	Gly	Tyr	Gly	Thr	Asp	Phe	Thr	Phe	Thr	Ile	Ser	Thr	Val	Gln	
	65					70					75				80		
	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Phe	Cys	Gln	Gln	Asp	Tyr	Arg	Ser	Pro	
				85						90				95			
20	Phe	Thr	Phe	Gly	Ser	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Val	Asp	Lys	Ser	
				100					105					110			
	Ser	Gly	Ser	Gly	Ser	Glu	Ser	Lys	Ser	Thr	Gln	Val	Lys	Leu	Glu	Glu	
			115					120					125				
	Ser	Gly	Pro	Gly	Ile	Leu	Gln	Pro	Ser	Gln	Thr	Leu	Ser	Leu	Thr	Cys	
25			130				135					140					
	Ser	Phe	Ser	Gly	Phe	Ser	Leu	Ser	Thr	Ser	Gly	Met	Gly	Val	Ser	Trp	
	145					150					155				160		
	Ile	Arg	Gln	Pro	Ser	Gly	Lys	Gly	Leu	Glu	Trp	Leu	Ala	His	Ile	Tyr	
				165					170					175			
30	Trp	Asp	Asp	Asp	Lys	His	Tyr	Asn	Pro	Ser	Leu	Lys	Ser	Arg	Leu	Thr	
				180					185					190			
35	Ile	Ser	Lys	Asp	Thr	Ser	Thr	Asn	Gln	Val	Phe	Leu	Lys	Ile	Thr	Ser	
			195					200					205				
	Val	Asp	Thr	Ala	Asp	Thr	Ala	Thr	Tyr	Phe	Cys	Ala	Arg	Arg	Ser	Leu	
		210					215					220					
	Tyr	Gly	Asn	Trp	Gly	Asp	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	
40		225				230					235				240		
	Ser	Val	Thr	Val	Ser	Ser											
						245											

(b) a polypeptide which in respect to (a) is deficient in one or more amino acids;

(c) a polypeptide in which in respect to (a) one or more amino acids are replaced;

(d) a fusion peptide comprising a polypeptide according to (a), (b) or (c) in which the additively connected amino acids do not interfere with the inhibition activity of the polypeptide or may be easily eliminated;

(e) a polypeptide in which in respect to (a) one or more amino acids are chemically modified.

The present invention further relates to a DNA-sequence coding for a polypeptide being capable of inhibiting binding of IL-6 to IL-6 receptor. Specifically the present invention is directed to a DNA-sequence being selected from the group consisting of:

(a) a DNA-sequence having the following nucleotide sequence;

5 ATG GAC ATC CTG CTG ACA CAG TCT CCA AAA TTC CTG CTT GTA TCA GCA 48
 GGA GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT ACT 96
 GAT GTA AGT TGG TAC CAA CAG AAG CCA GGG CAG TCT CCT AAA CTA CTG 144
 10 ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC TTC ACT 192
 GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG 240
 GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGG TCT CCA 288
 TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA GTC GAC AAA TCC 336
 15 TCA GGA TCT GGC TCC GAA TCC AAA AGC ACG CAG GTC AAA CTC GAG GAG 384
 TCT GGC CCT GGG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT TGT 432
 TCT TTC TCT GGG TTT TCA CTG AGC ACT TCT TGT GGT ATG GGT GTG AGC TGG 480
 ATT CGT CAG CCT TCA GGG AAG GGT CTG GAG TGG CTG GCA CAC ATT TAT 528
 TGG GAT GAT GAC AAA CAC TAT AAC CCA TCC CTG AAG AGC CGG CTC ACA 576
 20 ATC TCC AAG GAT ACC TCC ACC AAC CAG GTA TTC CTC AAG ATC ACC AGT 624
 GTG GAC ACT GCA GAT ACT GCC ACA TAC TTC TGT GCT CGA AGA AGT CTC 672
 TAT GGT AAT TGG GGG GAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC 720
 TCA GTC ACC GTC TCC TCA 738

- 20 (b) a DNA-sequence which in respect to (a) is deficient in one or more nucleotides;
 (c) a DNA-sequence in which in respect to (a) one or more nucleotides are replaced;
 (d) a DNA-sequence to which in respect to (a) one or more nucleotides are added.

The present invention further relates to an human IL-6 suppressant, to an anti-inflammatory agent, to an immunosuppressant or an agent for autoimmune disease treatment containing the above peptide, a gene
 25 which codes for the above peptide, a plasmid carrying the above gene, a transformant carrying the above plasmid, and a method for the production of the above peptide by culturing the above transformant.

The peptide according to the present invention specifically inhibits the binding of human IL-6 to human IL-6 receptor to suppress the function of IL-6, and it may be used as a treatment for autoimmune diseases such as rheumatoid arthritis, and systemic lupus erythematosus, a treatment for bacterial infections, a
 30 treatment for septic shock due to bacterial infections, a treatment for viral infections, a treatment for cancers such as multiple myeloma, a cancer metastasis suppressant, a medicine for the amelioration of cancer cachexia, and a treatment for inflammatory diseases such as mesangial proliferative glomerulonephritis. The ranges of use of the above are included in the terms human IL-6 suppressant, anti-inflammatory agent, immunosuppressant and autoimmune disease treatment, used in the present specification. A detailed
 35 description of the present invention will now be provided.

Monoclonal antibodies which inhibit the binding of IL-6 to IL-6 receptors by binding to IL-6 or to IL-6 receptors are useful as binding inhibitors with extremely high specificity, but in general the monoclonal antibodies for molecules in humans are foreign proteins obtained by immunizing animals other than humans, such as mice. Therefore, when monoclonal antibodies are administered to the human body,
 40 antibodies are produced against the administered antibodies, and an immune reaction by way of phagocytes, the complement system, etc. is brought about, raising the possibility of shock symptoms known as allergy, serum sickness, and the like.

An antibody molecule is known to consist of variable regions (hereunder abbreviated to V regions) which contribute directly to the binding to the antigen, and constant regions (hereunder abbreviated to C regions) which perform the function of binding to phagocytes for their activation, activating complement, and giving rise to various immune reactions. Of these regions, the C regions constitute approximately 2/3 of the antibody molecule, and generally when they are administered to an animal of a different species, antibodies tend to be produced.

On the other hand, the inhibition of the binding of IL-6 to IL-6 receptors by anti-IL-6 antibody or anti-IL-6
 50 receptor antibody requires the antigen-binding V regions of the antibody molecular structure, but the C regions of the antibody are not necessarily required. When the antibody containing the C regions is administered to the body, as described above, antibodies are produced against the antibody and form immune complexes with the antigen flowing in the blood, which can become trapped in the kidneys, etc. causing inflammation, or can bind with and be incorporated into phagocytes such as macrophages and the
 55 like, stimulating the release of inflammatory cytokines, chemical mediators, enzymes, etc. which further aggravate the inflammation. Therefore, it is preferred that the C regions not be present.

Antibody molecules assume a heterodimer structure in which H-chains (heavy chains) and L-chains (light chains) are bound to each other in the C regions via S-S bonds, and the H-chains are bound to each

other in the C regions via S-S bonds, and therefore in order to produce an antibody molecule consisting of the V regions alone, there may be imagined a method whereby the V regions of the H-chains and L-chains of the antibody molecule are obtained by restrictive digestion thereof using a proteolytic enzyme, after which they are bound together by some method to prepare a V region molecule consisting solely of a functional antibody. However, with the present technology it has not yet been possible to efficiently and easily produce molecules consisting solely of the functional antibody V regions.

Here, we, the present inventors have carried out a careful screening of monoclonal antibodies which are suitable for the following method, and, designing a single-chain peptide constructed from the V regions of the H-chains and L-chains of a few of the monoclonal anti-human IL-6 antibodies with a peptide linker using gene recombination, have invented an efficient method for the production of a peptide which specifically inhibits the binding of IL-6 to IL-6 receptor, possesses activity to suppress the function of IL-6, and has a clear medicinal effect.

In other words, first various types of hybridoma clones were prepared which produced mouse monoclonal antibodies which specifically bound to human IL-6 and inhibited the binding of human IL-6 and human IL-6 receptor, hence possessing activity to suppress the function of IL-6. The method for the preparation of the hybridoma clones producing mouse monoclonal anti-human IL-6 antibody is described below.

The hybridomas are prepared by fusing myeloma cells and antibody-producing cells. The antibody-producing cells may be spleen or lymph node cells from animals such as mice or rats which have been immunized with purified natural human IL-6 or recombinant human IL-6 produced using procaryotic cells such as *Escherichia coli* or the like, or eucaryotic cells such as yeast, animal cells, or the like.

The animal species from which the antibody-producing cells and myeloma cells are derived may be two different ones so long as both types of cells are fusible, but usually a more favorable result is obtained by using cells from the same species. One preferred hybridoma for carrying out the present invention is a hybridoma of spleen cells or lymph node cells from mice which have been immunized with recombinant human IL-6 produced by *E. coli*, with mouse myeloma cells.

For example, excellent results such as those demonstrated in the Examples which follow were obtained using hybridomas between spleen cells of Balb/c mice which were immunized with recombinant human IL-6 with Freund's complete adjuvant, and myeloma X63-Ag8-6.5.3 cells established from Balb/c mice.

The myeloma cells may be, in addition to X63-Ag8-6.5.3, 8-azaguanine-resistant cell strains, including SP2/0-Ag14, P3-X63-Ag8-U1, P3-X63-Ag8, P3-NSI/1-Ag4-1, MPC11-4.5.6.TG.1.7 (all mouse cells), 210.RCY.Ag1.2.3 (rat cells), SK0-007, GH15006TG-A12 (both human cells), etc.

The preparation of the hybridomas, and the selection therefrom of clones which produce monoclonal antibodies capable of binding to IL-6 and inhibiting the binding of IL-6 to IL-6 receptor for the suppression of the function of IL-6 may be carried out, for example, in the following manner. The antibody-producing cells and the myeloma cells are fused together using polyethylene glycol or the Sendai virus, etc. Only the fused hybridomas are capable of growth in a culture medium containing hypoxanthine, thymidine and aminopterin (HAT medium). Not all of the resulting hybridomas produce antibodies, and not all of the antibody-producing hybridomas produce the desired antibody, and therefore it is necessary to select those hybridomas producing monoclonal antibodies which are capable of binding to IL-6 and inhibiting the binding of IL-6 to IL-6 receptor for the suppression of the function of IL-6.

The selection may be made using, for example, the following method. That is, as the primary screening, a human-IL-6-coated plate is prepared and the hybridoma culture supernatant is reacted, and then 125 I-labelled anti-mouse IgG is reacted, and those hybridomas with high binding amounts thereto are selected. As the secondary screening, the inhibiting activity against the binding of 125 I-labelled IL-6 to IL-6 receptor-expressing cells and the inhibiting activity against the growth of cells whose growth is IL-6-dependent may be measured for the hybridoma culture supernatant, and those hybridomas with high levels of inhibiting activity selected.

The total RNA is extracted from the hybridoma clones obtained in this manner, and the gene (cDNA) coding for the V regions of the monoclonal antibody is obtained. The present inventors have diligently devised a method to quickly obtain the object cDNA, and thus the cDNA which codes for the V regions of the antibody has been obtained in the following manner.

That is, first, nucleotide sequences of 20-30 bases (primer DNA) with a high degree of correspondence to the 5' end and 3' end, respectively, of the gene coding for the V regions, are designed based on the nucleotide sequence for the H-chains and L-chains of mouse IgG, whose genetic nucleotide sequences have already been published.

Here, the 5' end primer is designed in the 5'→3' direction, and a translation initiation codon, ATG, is attached at the 5' end of the L-chain V region primer. For the 3' end primer, a translation termination codon

5 ATG GAC ATC CTG CTG ACA CAG TCT CCA AAA TTC CTG CTT GTA TCA GCA 48
 GGA GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT ACT 96
 GAT GTA AGT TGG TAC CAA CAG AAG CCA GGG CAG TCT CCT AAA CTA CTG 144
 ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC TTC ACT 192
 GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG 240
 GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGG TCT CCA 288
 TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA GTC GAC AAA TCC 336
 TCA GGA TCT GGC TCC GAA TCC AAA AGC ACG CAG GTC AAA CTC GAG GAG 384
 10 TCT GGC CCT GGG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT TGT 432
 TCT TTC TCT GGG TTT TCA CTG AGC ACT TCT GGT ATG GGT GTG AGC TGG 480
 ATT CGT CAG CCT TCA GGG AAG GGT CTG GAG TGG CTG GCA CAC ATT TAT 528
 TGG GAT GAT GAC AAA CAC TAT AAC CCA TCC CTG AAG AGC CGG CTC ACA 576
 ATC TCC AAG GAT ACC TCC ACC AAC CAG GTA TTC CTC AAG ATC ACC AGT 624
 15 GTG GAC ACT GCA GAT ACT GCC ACA TAC TTC TGT GCT CGA AGA AGT CTC 672
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other in the C regions via S-S bonds, and therefore in order to produce an antibody molecule consisting of the V regions alone, there may be imagined a method whereby the V regions of the H-chains and L-chains of the antibody molecule are obtained by restrictive digestion thereof using a proteolytic enzyme, after which they are bound together by some method to prepare a V region molecule consisting solely of a functional antibody. However, with the present technology it has not yet been possible to efficiently and easily produce molecules consisting solely of the functional antibody V regions.

Here, we, the present inventors have carried out a careful screening of monoclonal antibodies which are suitable for the following method, and, designing a single-chain peptide constructed from the V regions of the H-chains and L-chains of a few of the monoclonal anti-human IL-6 antibodies with a peptide linker using gene recombination, have invented an efficient method for the production of a peptide which specifically inhibits the binding of IL-6 to IL-6 receptor, possesses activity to suppress the function of IL-6, and has a clear medicinal effect.

In other words, first various types of hybridoma clones were prepared which produced mouse monoclonal antibodies which specifically bound to human IL-6 and inhibited the binding of human IL-6 and human IL-6 receptor, hence possessing activity to suppress the function of IL-6. The method for the preparation of the hybridoma clones producing mouse monoclonal anti-human IL-6 antibody is described below.

The hybridomas are prepared by fusing myeloma cells and antibody-producing cells. The antibody-producing cells may be spleen or lymph node cells from animals such as mice or rats which have been immunized with purified natural human IL-6 or recombinant human IL-6 produced using procaryotic cells such as *Escherichia coli* or the like, or eucaryotic cells such as yeast, animal cells, or the like.

The animal species from which the antibody-producing cells and myeloma cells are derived may be two different ones so long as both types of cells are fusible, but usually a more favorable result is obtained by using cells from the same species. One preferred hybridoma for carrying out the present invention is a hybridoma of spleen cells or lymph node cells from mice which have been immunized with recombinant human IL-6 produced by *E. coli*, with mouse myeloma cells.

For example, excellent results such as those demonstrated in the Examples which follow were obtained using hybridomas between spleen cells of Balb/c mice which were immunized with recombinant human IL-6 with Freund's complete adjuvant, and myeloma X63-Ag8-6.5.3 cells established from Balb/c mice.

The myeloma cells may be, in addition to X63-Ag8-6.5.3, 8-azaguanine-resistant cell strains, including SP2/0-Ag14, P3-X63-Ag8-U1, P3-X63-Ag8, P3-NS1/1-Ag4-1, MPC11-4.5.6.TG.1.7 (all mouse cells), 210.RCY.Ag1.2.3 (rat cells), SK0-007, GH15006TG-A12 (both human cells), etc.

The preparation of the hybridomas, and the selection therefrom of clones which produce monoclonal antibodies capable of binding to IL-6 and inhibiting the binding of IL-6 to IL-6 receptor for the suppression of the function of IL-6 may be carried out, for example, in the following manner. The antibody-producing cells and the myeloma cells are fused together using polyethylene glycol or the Sendai virus, etc. Only the fused hybridomas are capable of growth in a culture medium containing hypoxanthine, thymidine and aminopterin (HAT medium). Not all of the resulting hybridomas produce antibodies, and not all of the antibody-producing hybridomas produce the desired antibody, and therefore it is necessary to select those hybridomas producing monoclonal antibodies which are capable of binding to IL-6 and inhibiting the binding of IL-6 to IL-6 receptor for the suppression of the function of IL-6.

The selection may be made using, for example, the following method. That is, as the primary screening, a human-IL-6-coated plate is prepared and the hybridoma culture supernatant is reacted, and then ¹²⁵I-labelled anti-mouse IgG is reacted, and those hybridomas with high binding amounts thereto are selected. As the secondary screening, the inhibiting activity against the binding of ¹²⁵I-labelled IL-6 to IL-6 receptor-expressing cells and the inhibiting activity against the growth of cells whose growth is IL-6-dependent may be measured for the hybridoma culture supernatant, and those hybridomas with high levels of inhibiting activity selected.

The total RNA is extracted from the hybridoma clones obtained in this manner, and the gene (cDNA) coding for the V regions of the monoclonal antibody is obtained. The present inventors have diligently devised a method to quickly obtain the object cDNA, and thus the cDNA which codes for the V regions of the antibody has been obtained in the following manner.

That is, first, nucleotide sequences of 20-30 bases (primer DNA) with a high degree of correspondence to the 5' end and 3' end, respectively, of the gene coding for the V regions, are designed based on the nucleotide sequence for the H-chains and L-chains of mouse IgG, whose genetic nucleotide sequences have already been published.

Here, the 5' end primer is designed in the 5'→3' direction, and a translation initiation codon, ATG, is attached at the 5' end of the L-chain V region primer. For the 3' end primer, a translation termination codon

is attached at the 3' end of the primer for the H-chain, that is, the chain for which no ATG sequence has been attached. In the case of the 3' end primer, the complementary sequence may be redesigned in the 3'→ 5' direction.

Naturally, an ATG sequence may be attached at the 5' end of the H-chain V region primer, and a termination codon attached at the 3' end of the L-chain V region primer. As the termination codon may be used TAA, TAG or TGA. In the Examples according to the present invention, TGA was used as the termination codon.

Next, an appropriate restriction enzyme site is introduced at the 5' end of the primer DNA of the respective H-chains and L-chains (in the case of the 3' end primer, at the 5' end of the redesigned primer DNA), for insertion into an expression vector. The designed primer DNA is chemically synthesized using a DNA synthesizer or the like.

Further, the total RNA is extracted from the resulting hybridomas by a conventional method, single-stranded cDNA is prepared using reverse transcriptase and the 3' end primer DNA, and then, using the 5' end primer DNA and the 3' end primer DNA, the DNA fragments coding for the V regions of the H-chain and L-chain of the antibody are obtained by their selective amplification by the Polymerase Chain Reaction (PCR method, Science, Vol.230, p.1350, 1985) using Taq polymerase.

When the obtained genes coding for these V regions are expressed in *E. coli* to prepare functional antibody V region molecules, the respective genes may be incorporated separately or simultaneously into a single vector, and then assembled for expression, but the efficiency of this method is known to be extremely poor (Science, Vol.240, p.1038, 1988).

Recently, a technique has been developed whereby the V regions are linked together using a linker, and are thus expressed in *E. coli* as a single chain molecule (Science, Vol.242, p.423, 1988). Here, we, the present inventors, have applied this technique, and through further manipulation and improvement, have succeeded in expressing a single peptide chain consisting of the V regions of human IL-6 antibody, and in maintaining and even improving the properties with no loss of the function to inhibit the binding of human IL-6 to human IL-6 receptor.

In other words, first, an expression vector is constructed to include, in the following order from upstream, a promoter, a ribosome-binding site, a restriction enzyme site which has been introduced into the 5' end primer with an ATG sequence attached thereto, a restriction enzyme site which has been introduced into the 3' end primer of the same chain, a DNA sequence coding for a linker peptide of suitable length for joining the V region of the antibody L-chains and the V region of the antibody H-chains, a restriction enzyme site which has been introduced into the 5' end primer with no ATG sequence attached thereto, a restriction enzyme site which has been introduced into the 3' end primer of the same chain, and finally a terminator.

Here, when introducing the DNA of each V region which was amplified by the PCR, care should be taken so that the translation of the linker peptide and the following V region DNA is not shifted.

For the present invention, the source from which the promoter is derived is not critical, and for example, trp promoter, tac promoter, trc promoter, lac promoter from *E. coli* and λ P_L promoter, λ P_R promoter from λ -phage, etc. may be used. The ribosome-binding site to be used may be the ribosome-binding site of trpL, trpE or lacZ from *E. coli* or of CII protein from λ -phage. Alternatively, a chemically synthesized DNA sequence may be used. In addition, for a large accumulation of the object peptide in a granular form in *E. coli*, 2 or more ribosome-binding sites may be employed.

The linker peptide sequence for joining the respective antibody V regions together may be any one so long as the prepared antibody V region molecule is functional, but in order to minimize the side effects of the molecule when administered to the human body, the sequence is preferably as short as possible and with no unique structure. Furthermore, manipulation is required to ensure that, once the peptide is expressed, the function of the V regions is not lost or attenuated. The terminator to be used may be, for example, trpA terminator, rrnB terminator, recA terminator, etc. from *E. coli*. Also, usually a larger number of copies of the expression plasmid is preferred, and the replication origin is preferred to be pUC rather than pBR.

The PCR-amplified DNA for each of the H-chain and L-chain V regions is inserted into the constructed expression plasmid. After the insertion, the expression plasmid may be used to transform a host by a conventional method, and then expressed. The host may be any procaryotic or eucaryotic organism. Examples of procaryotic organisms include *E. coli*, *Bacillus subtilis*, etc. The eucaryotic cells to be used may be, for example, yeast, CHO cells, or the like. Suitable expression vectors and shuttle vectors for procaryotic and eucaryotic cells are well known in the art and are described e.g. in Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press 1989 (T. Maniatis et al.), which document is included herein as reference. Based on common knowledge and the information provided

herein the person skilled in the art will select appropriate regulatory sequences, such as e.g. specific promoters, terminators and/or enhancers for constructing expression vectors for the particular host to be used. For the purpose of easily expressing the desired polypeptide procaryotic cells, and particularly E. coli, are preferred for use as the host.

5 The method of incorporating the expression vector into these hosts may be any conventional method; for example, one in which E. coli cells in the logarithmic growth phase are treated with 50 mM of calcium chloride on ice for about 30 minutes to modify the structure of the cell wall of E. coli, and then 10 minutes after the plasmid DNA is injected therein the cells are subjected to heat treatment at 30-42°C for 2 minutes, after which a culture medium is added thereto for culturing at 30-37°C for about 60 minutes, to
10 incorporate the expression plasmid DNA into the organism.

By culturing these cells, the object peptide which inhibits the binding of human IL-6 to human IL-6 receptor, particularly binding of human IL-6 to human IL-6 receptor may, be accumulated either in the cells themselves or in the culture medium. The medium may be any of a number of publicly known ones in which the culturing of cells is possible, and the culturing conditions may be any publicly known ones. After
15 the culturing, the object peptide may be recovered by a conventional method.

The peptide according to the present invention possesses activity to inhibit the binding of human IL-6 to human IL-6 receptor for the suppression of the function of human IL-6, and thus it is a effective substance for use as a treatment for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, a treatment for bacterial infections, a treatment for septic shock due to bacterial infections, a
20 treatment for viral infections, a treatment for cancers such as multiple myeloma, a cancer metastasis suppressant, a medicine for the amelioration of cancer cachexia, and a treatment for inflammatory diseases such as mesangial proliferative glomerulonephritis, and other diseases thought to be induced or aggravated by IL-6.

Furthermore, the structure of the peptide according to the present invention is not limited to the sequence listed as Sequence No. 1 in the Sequence List, as the peptide according to the present invention
25 includes any peptide which possesses activity to inhibit the binding of human IL-6 to human IL-6 receptor for the suppression of the function of human IL-6. For example, included in the peptide according to the present invention are (1) a linear or cyclic peptide lacking one or more amino acids from the N-terminus and/or C-terminus of the peptide structure listed as Sequence No. 1 and has a continuous amino acid
30 sequence, (2) a peptide having a structure wherein one or more amino acids in the peptide structure listed as Sequence No. 1 are replaced with other amino acids, (3) a peptide having a structure wherein one or more amino acids are added at the N-terminus and/or C-terminus of the peptide structure listed as Sequence No. 1, and (4) a peptide having a structure wherein one or more amino acids of the peptide listed
35 as Sequence No. 1 are acetylated, amidized or polyethylene glycol-added, so long as the peptide possesses activity to inhibit the binding of human IL-6 to human IL-6 receptor for the suppression of the function of human IL-6.

In particular, the N-terminal Met of the peptide listed as Sequence No. 1 in the Sequence List is sometimes cut off during the process of expression by a microorganism or purification, leaving Asp at the N-terminus. Such a peptide is also included in the peptide according to the present invention so long as it
40 possesses the activity mentioned above. In addition, a peptide prepared by removing the N-terminal Met from the resulting Met-bonded peptide using amino peptidase or the like is also included in the peptide according to the present invention so long as it possesses the activity mentioned above.

Furthermore, peptides prepared by converting the so-called framework regions of the peptides according to the present invention into human-derived frameworks are naturally expected to possess the same
45 activity as the peptides prior to the conversion, and thus they are also included in the peptide according to the present invention, so long as they possess the activity mentioned above.

In addition, if necessary a peptide prepared by adding a toxin to the peptide according to the present invention may be used.

An immunosuppressant according to the present invention contains the above mentioned peptide at a
50 proportion of 0.1-100 wt %, or preferably 0.5-70 wt %. Therefore, the peptide according to the present invention may be administered directly or in the form of a preparation thereof made by mixing it with a common preparation carrier. The preparation carrier to be used may be any commonly used substance which does not react with the peptide according to the present invention. In the case of an injection, the peptide according to the present invention is prepared for use by dissolution in water, and if necessary it
55 may be dissolved in physiological saline or a glucose solution, and it may contain a buffering agent, preservative, stabilizer or diluent. In addition, these preparations may also contain other components with therapeutic value.

The method for administration of the immunosuppressant according to the present invention may be oral, by injection, intrarectal, etc., but administration by injection is preferred. The dosage will differ depending upon the method of administration and the symptoms, age, etc. of the patient, but normally it is 0.001-1000 mg for a single dose, and preferably 0.01-10 mg administered 1-3 times per day.

EXAMPLES

A more detailed description of the present invention will now be provided with reference to the Examples, but the present invention is not limited to these Examples.

Example 1 (Preparation of hybridomas)

Six to 8-week-old female BALB/c mice were immunized by subcutaneous administration of 100 μ g/mouse of recombinant human IL-6 with Freund's complete adjuvant. Three weeks afterwards, they were subjected to booster immunization in the same manner, and then 5 days afterwards blood was taken from the orbital vein. Recombinant human IL-6 (2 μ g/ml) dissolved in phosphate buffered saline (PBS) was separately placed in the wells of 96-well microtiter plates (product of Flow Co.) and left to stand overnight at 4°C. The plates were then treated with PBS containing 0.5% bovine serum albumin (BSA-PBS) and incubated for 1 hr at room temperature. Sample sera diluted to an appropriate concentration with BSA-PBS were added to each well and incubated for 2 hr at room temperature. After washing the plates with PBS containing 0.05 % Tween-20 (product of Nacalai tesque Co.) (PBS-Tween), anti mouse Ig antibody (product of Daco Co.) was added to each well, followed by incubation for 2 hr at room temperature. The plates were then washed with PBS-Tween and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfuric acid) (ABTS; product of Sigma Co.) and hydrogen peroxide were added to each well and the absorbance (A405) of the wells were measured. The IL-6-binding activity thereof was tested to determine the antibody titer. Mice with a high antibody titer were further subjected to a final immunization in the same manner, and 3 days afterwards the spleens were extracted, and the spleen cells and mouse myeloma cells (X63-Ag8-6.5.3) were mixed together in the presence of 50% polyethylene glycol #4000 (product of Nacalai tesque Co.) to a cell count proportion of 10:1, for fusion of the cells.

The fused cells were suspended in an RPMI1640 culture medium (product of Gibco Co.) containing 10% bovine calf serum (product of Gibco Co.) to a cell concentration of 5×10^6 cells/ml, and a 100 μ l portion thereof was poured into each well of a 96-well flat-bottom plate (product of Corning Co.) which contained 5×10^5 mouse thymus cells per well. At 1, 2, 3 and 6 days afterwards, half of the culture medium was replaced with a medium containing hypoxanthine, aminopterin and thymidine (HAT medium), and the process was repeated every 3 days thereafter. The culture supernatant of each well in which the fused cells (hybridomas) had grown was tested, and those hybridomas in the wells which possessed IL-6-binding activity were cloned by the limiting dilution method.

In addition, the IL-6-binding activities of culture supernatants of each of the hybridoma clones were determined, and the anti-IL-6 antibody-producing hybridomas were obtained. Further, the culture supernatants of the obtained anti-IL-6 antibody-producing hybridomas were tested for suppression against the function of IL-6, using the following method. A 50 μ l portion of a human recombinant IL-6 solution prepared with a concentration of 200 pg/ml in an RPMI1640 culture medium containing 10% bovine calf serum was poured into each well of a 96-well flat-bottom microplate, 50 μ l of one of the sample culture supernatants was added thereto, and the cells were incubated at 37°C for 30 minutes. Also, 100 μ l of an MH60 cell suspension in an RPMI1640 culture medium containing 10% bovine calf serum to a concentration of 1×10^5 cells/ml was added to each well, and the cells were cultured at 37°C for 3 days, in the presence of 5% CO₂. For the final 6 hours, the culturing was carried out with addition thereto of 1 μ Ci of 3H-thymidine (product of DuPont Co.), and the amount of radioactivity taken into the cells was measured using a scintillation counter (product of Packard Co.) to determine the activity of suppression against the function of IL-6 for the culture supernatant. Thus were prepared hybridomas producing antibodies against IL-6. One of the hybridomas obtained in this manner is HH61-10 (see Japanese Patent Application Disclosure HEI 3-61496).

Example 2 (Preparation of antibody V region cDNA from the hybridomas)

Of the hybridoma HH61-10 cells, 5×10^6 were washed with PBS, and an RNA extraction buffer solution (product of Pharmacia Co.) containing guanidine thiocyanate, N-lauryl sarcosine and EDTA was added thereto to prepare a suspension, which was then overlaid on an equivolume of caesium chloride solution

($\rho = 1.51$ g/ml, product of Pharmacia Co.) provided beforehand in a tube and subjected to centrifugation at 125,000 x g for 16 hours. After the supernatant thereof was sucked off, a 10 mM Tris-HCl buffer solution containing 1 mM of EDTA (pH 7.5) was added to the residue to prepare a suspension, which was poured into a fresh tube, for incubation at 65°C for 5 minutes. In addition, a 1/10 volume of 2 M potassium acetate (pH 5.0) (product of Pharmacia Co.) and a 3-fold volume of ethanol (product of Nacalai tesque Co.) were added thereto, and the mixture was allowed to stand at -20°C overnight. It was then subjected to centrifugation at 5,000 x g for 20 minutes and the supernatant thereof discarded, after which the residue was subjected to centrifugal washing with 80% ethanol, and the resulting precipitate was dried. The precipitate was dissolved in a 10 mM Tris-HCl buffer solution containing 1 mM of EDTA (pH 7.5) to prepare a solution of the total RNA fraction.

Next, to the total RNA fraction solution were added a solution containing a predesigned primer complementary to the 3' end of the gene for the V regions of the H-chains and L-chains of the antibody (final concentration: 1 μ M), a mixed solution of deoxyNTP, a buffer solution for cDNA synthesis (product of Amersham Co.), RNase inhibitor (product of Takara Shuzou Co.) and reverse transcriptase (product of Takara Shuzou Co.), and these were reacted at 42°C for 1 hour to synthesize the cDNA. In addition, a PCR buffer solution (product of Cetus Co.), a mixed solution of deoxyNTP, 5' end primer and 3' end primer for amplification of the V regions of the H-chains and L-chains of the antibody (both final concentrations: 1 μ M) and Taq polymerase (product of Takara Shuzou) were added thereto for the PCR (thermal cycler, product of Cetus Co.). The reaction was carried out with 30 cycles each consisting of denaturing for 30 seconds (94°C), annealing for 30 seconds (55°C) and primer extension for 1 minute (72°C), and each cycle the primer extension time was lengthened by 15 seconds.

After the reaction, the product was subjected to agarose gel electrophoresis in a 40 mM Tris-HCl-acetate buffer solution containing 1 mM of EDTA (pH 8.0), and the object cDNA fragments were cut out and then extracted and purified using a gene clean kit (product of Bio 101 Co.). The sequences of the primers used for the synthesis of the cDNA and the PCR are shown in Fig. 1.

Example 3 (Construction of the expression vector)

As shown in Fig. 3, first the large DNA fragments obtained by cleaving pT13SNco (*E. coli* AJ-12447 containing this plasmid has been deposited as FERM P-10757) [listed in J. Biochem., Vol.104, p.30, 1988] with restriction enzymes ClaI and BamHI (both products of Takara Shuzou Co.) and a conventionally prepared synthesized DNA fragment (linker) having the sequence shown in Fig. 2, were linked using T4DNA ligase (product of Takara Shuzou Co.).

Next, also as shown in Fig. 3, the synthesized DNA fragment was linked with the larger fragment obtained by cleaving the plasmid pT13SNco with restriction enzymes ClaI and BamHI. A plasmid pFv-DE having a pUC origin of replication was obtained by using T4 ligase to ligate the smaller DNA fragment obtained by cleaving the previously mentioned linked plasmid with restriction enzymes EcoRI and PvuII (both products of Takara Shuzou Co.) with the larger DNA fragment obtained by using EcoRI and HindII (products of Takara Shuzou Co.) to cleave plasmid pUC18 (Methods in Enzymology, Vol.101, p.20, 1983) whose HindIII and NdeI sites had been removed by cleavage with the corresponding restriction enzymes and then making the ends blunt with T4 DNA polymerase (product of Takara Shuzou Co.), and finally ligating them with T4 ligase.

Example 4 (Insertion of antibody V region cDNA into pFv-DE and preparation of a *E. coli* strain producing antibody consisting solely of the V regions)

As shown in Fig. 4, first T4 ligase was used to ligate the larger DNA fragment obtained by cleaving pFv-DE with restriction enzymes NdeI and SalI (products of Takara Shuzou Co.) with the fragment obtained by cleaving the L-chain V region cDNA of HH61-10 recovered after the PCR using the same restriction enzymes NdeI and SalI. The L-chain V region of HH61-10 corresponds to the 4th-324th bases of the nucleotide sequence listed as Sequence No. 1 in the Sequence List. In both sequences, the 1st-3rd bases of the sequence are the translation initiation codon ATG.

Next, T4 ligase was used to ligate the larger DNA fragment obtained by further cleaving the above ligated plasmid with restriction enzymes XhoI and HindIII (products of Takara Shuzou Co.) with the fragment obtained by cleaving the H-chain V region cDNA of HH61-10 recovered after the PCR using the same restriction enzymes XhoI and HindIII, in order to construct and obtain a plasmid pFv (HH61-10)-DE expressing an antibody consisting solely of the V regions. Next, each of the plasmids was used to transform *E. coli* HB101 strain to obtain a strain producing antibody consisting solely of the V regions (*E. coli* pFv

(HH61-10)-DE/HB101, AJ-12789, FERM BP-4523).

The H-chain V region of HH61-10 corresponds to the 367th-738th bases of the nucleotide sequence listed as Sequence No. 1 in the Sequence List.

5 Example 5 (Acquisition of product from the strain producing antibody consisting solely of the V regions)

The resulting transformant *E. coli* pFv (HH61-10)-DE/HB101 (FERM BP-4523) was grown overnight at 37 °C in 5 ml of 2 x YT [1.6 % tryptone, 1% yeast extract (both products of Bacto Co.), 0.5% NaOH, pH 7.0] containing 50 µg/ml of ampicillin. Next, 5 ml of the culture suspension was seeded into 100 ml of an
 10 M9-casamino acid culture medium (0.6% Na₂HPO₄ · 12H₂O, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.05% MgSO₄ · 7H₂O, 0.00147% CaCl₂, 0.2% glucose, 0.2% of casamino acid, 0.02% L-leucine, 0.02% L-proline, 0.0002% thiamine hydrochloride, 100 µg/ml ampicillin, pH 6.9), for culturing at 37 °C for 3 hours. Following this, 3-indoleacrylic acid (IAA) was added thereto to a concentration of 25 µg/ml, and induction culturing was continued at 37 °C for 20 hours. A portion of this cell suspension was observed using a phase
 15 contrast microscope at about 1500 x magnification, and inclusion body formation was found in the *E. coli* cells.

Next, the cell suspension which was cultured in the manner described above was subjected to centrifugal separation and the cells thereof collected and 50 ml of a 20 mM Tris-HCl buffer solution containing 30 mM of NaCl (pH 7.5) was added thereto to form a suspension, after which 12.5 ml of a
 20 solution of lysozyme in 0.5 M EDTA (pH 8.0) to a concentration of 1 mg/ml was added thereto and the mixture was stirred and then allowed to stand on ice for 1 hour. Next, the cells were disrupted ultrasonically, and then subjected to centrifugal separation at 6,000 rpm for 5 minutes, after which the inclusion bodies were collected. The inclusion bodies were solubilized with 6 M guanidine hydrochloride, and adjustment was made for a 100 µg/ml concentration of the object peptide and a 3.5 M guanidine hydrochloride
 25 solution, after which 3 µM of oxidized glutathione and 30 µM of reduced glutathione were added thereto, and the mixture was allowed to stand at a pH of 8.0, at room temperature for 10-16 hours. Then, the mixture was dialyzed against a 10 mM acetate buffer solution (pH 5.0) to obtain the crude peptide.

Next, the crude peptide was passed through an S-Sepharose column (product of Pharmacia Co.) which had been equilibrated in advance with a 10 mM acetate buffer solution (pH 5.0), and then eluted with a 10
 30 mM acetate buffer solution containing 50 mM of NaCl (pH 5.0), after which the eluate was dialyzed against PBS to obtain the object peptide.

The molecular weight of this substance determined according to SDS-polyacrylamide gel electrophoresis roughly matched the value obtained by calculation based on the amino acid sequence deduced in Example 6, and as a result of investigation of the N-terminal amino acid sequence using a protein
 35 sequencer, confirmation was made of the sequence having methionine added to the N-terminus of the deduced amino acid sequence.

Example 6 (Determination of the nucleotide sequence and deduction of the amino acid sequence)

40 The constructed plasmid pFv(HH61-10)-DE expressing the peptide consisting solely of the V regions was purified by the alkali SDS method, and commercially available M4 or RV primer for sequencing (products of Takara Shuzou Co.) were used to determine the nucleotide sequence with a 7-DEAZA sequencing kit (product of Takara Shuzou Co.). The amino acid sequence was deduced from the obtained
 nucleotide sequence.

45 The nucleotide sequence coding for the peptide Fv(HH61-10) and the amino acid sequence corresponding thereto are listed as Sequence No. 1 of the Sequence List. That is, as shown in Sequence No. 1 of the Sequence List, the peptide Fv(HH61-10) is a peptide comprising 246 amino acids, having Met at the N-terminus and Ser at the C-terminus.

50 Example 7 (Detection of the activity of peptide Fv(HH61-10))

(1) Inhibiting activity against binding of IL-6 to IL-6 receptor

The peptide Fv(HH61-10) diluted to an appropriate concentration with an RPMI1640 medium containing
 55 0.5% BSA and 0.02% NaN₃ was mixed with human recombinant IL-6 which had been ¹²⁵I-labelled by the Bolton-Hunter method, and these were reacted at room temperature for 1 hour. After the reaction, 5 x 10⁵ U266 cells suspended in the same medium were added thereto for reaction at 4 °C for one hour, and the radioactivity which had bound to the cells was measured. As shown in Fig. 5, it was clear that the peptide

Fv(HH61-10) possesses activity to inhibit the binding of human IL-6 to human IL-6 receptor.

(2) Inhibiting activity against growth of IL-6-dependent cell line

5 The peptide Fv(HH61-10) diluted to an appropriate concentration with an RPMI1640 medium containing 10% FCS was mixed with 0.4 ng/ml of human recombinant IL-6, and these were reacted at 37°C for 30 minutes. After the reaction, 5 x 10³ KT-3 cells suspended in the same medium were added thereto and cultured at 37°C for 2 days. After the culturing, an MTT reagent (product of Sigma Co.) prepared in the same medium to a concentration of 5 mg/ml was added thereto for further culturing at 37°C for 4 hours, 10 after which the cells were dissolved with isopropanol containing 0.04 N HCl, and the absorbance of the solution at 570 nm was measured to determine the viable cell count. As a result, as shown in Fig. 6, it was clear that the peptide Fv(HH61-10) possesses activity to inhibit the IL-6-dependent growth of KT-3 cells. That is, this peptide Fv(HH61-10) was shown to be a useful substance for the treatment of diseases such as multiple myeloma, rheumatoid arthritis, mesangial proliferative glomerulonephritis and the like, which are 15 caused by the IL-6-induced abnormal growth of cells.

(3) Inhibiting activity against acute phase protein production induced by IL-6

20 The peptide Fv(HH61-10) diluted to an appropriate concentration with a Dulbecco modified Eagle medium containing 10% FCS (product of Gibco Co.) was mixed with 100 ng/ml of human recombinant IL-6, and these were reacted at 37°C for 30 minutes. After the reaction, the mixture was reacted with Hep3B cells which had precultured in the same medium at 37°C for 16 hours at an initial population of 1 x 10⁴ cells per well, and these were further cultured at 37°C for 2 days. Next, each of the culture supernatants was taken out its well, and the haptoglobin contents thereof were assayed by the ELISA method. The ELISA 25 method comprised first coating sheep anti-human haptoglobin antibody (product of Binding Site Co.) dissolved in PBS to a concentration of 1 µg/ml into each well of a 96-well plate (product of Flow Co.) and reacting them at 4°C overnight. After the reaction, a PBS solution containing 0.5% BSA was added to the mixture which was then allowed to stand at room temperature for 1 hour. The PBS solution containing 0.5% BSA was discarded, and then each culture supernatant diluted 4-fold with the same type of solution was 30 added to the residue for reaction at room temperature for 2 hours. After the reaction, the solution was washed 3 times with a PBS solution containing 0.05% Tween20, and alkali phosphatase-labelled sheep anti-human haptoglobin antibody (product of Binding Site Co.) which had been diluted 1,000-fold with a PBS solution containing 0.5% BSA was added thereto, for reaction at room temperature for 2 hours. After the reaction, the solution was washed 3 times with a PBS solution containing 0.05% Tween20, p-nitrophenol 35 phosphate (product of Sigma Co.) adjusted to a concentration of 1 mg/ml with a 0.1 M carbonate buffer solution (pH 9.6) was added to the mixture which was then allowed to stand for 15 minutes, and then the absorbance at 405 nm was measured. As a result, as shown in Fig. 7, it was clear that the peptide Fv(HH61-10) possesses activity to inhibit the IL-6-induced production of haptoglobin, a type of acute phase protein, by Hep3B cells. That is, this peptide Fv(HH61-10) was shown to be a useful substance for the 40 treatment of inflammatory diseases.

EFFECT OF THE INVENTION

45 As mentioned above, the peptide according to the present invention possesses activity to inhibit the binding of human IL-6 to human IL-6 receptor for the suppression of the function of human IL-6, and thus it is a effective substance for use as a treatment for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, a treatment for bacterial infections, a treatment for septic shock due to bacterial infections, a treatment for viral infections, a treatment for cancers such as multiple myeloma, a cancer metastasis suppressant, a medicine for the amelioration of cancer cachexia, and a treatment for 50 inflammatory diseases such as mesangial proliferative glomerulonephritis, and other diseases thought to be induced or aggravated by IL-6.

BRIEF DESCRIPTION OF THE DRAWINGS

55 Fig. 1 shows a primer sequence.

Fig. 2 shows a linker DNA sequence for linking the V region of the L-chain and the V region of the H-chain.

Fig. 3 is a drawing showing a process for the construction of plasmid pFv-DE.

Fig. 4 is a drawing showing a process for the construction of plasmid pFv(HH61-10)-DE.

Fig. 5 is a drawing showing peptide Fv(HH61-10) possessing activity to inhibit the binding of ¹²⁵I-labelled IL-6 to U266 cells.

Fig. 6 is a drawing showing peptide Fv(HH61-10) possessing activity to inhibit the IL-6-dependent growth of KT-3 cells.

Fig. 7 is a drawing showing peptide Fv(HH61-10) possessing activity to inhibit the IL-6-dependent haptoglobin production by Hep3B cells.

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SEQUENCE LISTINGS

GENERAL INFORMATION:

Applicant

Name : Ajinomoto Co. Inc.
 Street : 15-1, Kyobashi 1 Chome
 City : Tokyo
 Country : Japan
 Postal Code : 104
 Telephone : (03) 5250-8111
 Fax : (03) 5250-8347 .
 Telex : J22690

TITLE OF THE INVENTION : Immunosuppressant

NUMBER OF SEQUENCES : 8

COMPUTER READABLE FORM

Medium type : Diskette
 Computer : IBM-PC compatible
 Operating system : MS-DOS

INFORMATION FOR SEQUENCE NO 1:

Sequence characteristics

length : 256 amino acids
 type : amino acid
 topology : linear
 molecule type : polypeptide

Met	Asp	Ile	Leu	Leu	Thr	Gln	Ser	Pro	Lys	Phe	Leu	Leu	Val	Ser	Ala
1				5					10					15	
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Ser	Val	Ser	Thr
			20					25					30		
Asp	Val	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys	Leu	Leu
		35					40				45				
Ile	Tyr	Tyr	Ala	Ser	Asn	Arg	Tyr	Thr	Gly	Val	Pro	Asp	Arg	Phe	Thr
		50				55					60				

Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Thr Val Gln
 65 70 75 80
 Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln Asp Tyr Arg Ser Pro
 85 90 95
 Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys Val Asp Lys Ser
 100 105 110
 Ser Gly Ser Gly Ser Glu Ser Lys Ser Thr Gln Val Lys Leu Glu Glu
 115 120 125
 Ser Gly Pro Gly Ile Leu Gln Pro Ser Gln Thr Leu Ser Leu Thr Cys
 130 135 140
 Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser Gly Met Gly Val Ser Trp
 145 150 155 160
 Ile Arg Gln Pro Ser Gly Lys Gly Leu Glu Trp Leu Ala His Ile Tyr
 165 170 175
 Trp Asp Asp Asp Lys His Tyr Asn Pro Ser Leu Lys Ser Arg Leu Thr
 180 185 190
 Ile Ser Lys Asp Thr Ser Thr Asn Gln Val Phe Leu Lys Ile Thr Ser
 195 200 205
 Val Asp Thr Ala Asp Thr Ala Thr Tyr Phe Cys Ala Arg Arg Ser Leu
 210 215 220
 Tyr Gly Asn Trp Gly Asp Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
 225 230 235 240
 Ser Val Thr Val Ser Ser
 245

25 INFORMATION FOR SEQUENCE NO 2:

Sequence characteristics

length : 738 base pairs
 type : nucleic acid
 strandedness : single
 topology : linear
 anti-sense : no

ATG GAC ATC CTG CTG ACA CAG TCT CCA AAA TTC CTG CTT GTA TCA GCA 48
 GGA GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT ACT 96
 GAT GTA AGT TGG TAC CAA CAG AAG CCA GGG CAG TCT CCT AAA CTA CTG 144
 ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC TTC ACT 192
 GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG 240
 GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGG TCT CCA 288
 TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA GTC GAC AAA TCC 336
 TCA GGA TCT GGC TCC GAA TCC AAA AGC ACG CAG GTC AAA CTC GAG GAG 384
 TCT GGC CCT GGG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT TGT 432
 TCT TTC TCT GGG TTT TCA CTG AGC ACT TCT GGT ATG GGT GTG AGC TGG 480
 ATT CGT CAG CCT TCA GGG AAG GGT CTG GAG TGG CTG GCA CAC ATT TAT 528
 TGG GAT GAT GAC AAA CAC TAT AAC CCA TCC CTG AAG AGC CGG CTC ACA 576

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ATC TCC AAG GAT ACC TCC ACC AAC CAG GTA TTC CTC AAG ATC ACC AGT 624
GTG GAC ACT GCA GAT ACT GCC ACA TAC TTC TGT GCT CGA AGA AGT CTC 672
TAT GGT AAT TGG GGG GAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC 720
TCA GTC ACC GTC TCC TCA 738

10 INFORMATION FOR SEQUENCE NO 3:

Sequence characteristics

length : 23 bases
15 type : nucleic acid
strandedness : single
topology : linear

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CAGGTSMARCTCGAGSAGTCWGG

25 INFORMATION FOR SEQUENCE NO 4:

Sequence characteristics

length : 32 bases
30 type : nucleic acid
strandedness : single
topology : linear

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AAGCTTCATGAGGAGACGGTGACCGTGGTCCC

40 INFORMATION FOR SEQUENCE NO 5:

Sequence characteristics

length : 45 bases
45 type : nucleic acid
strandedness : single
topology : linear

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ACAGTCATAATGTCCCATATGGAYATYCWGMTGACMCAGTCTCCA

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INFORMATION FOR SEQUENCE NO 6:

5 Sequence characteristics
length : 42 bases
type : nucleic acid
10 strandedness : single
topology : linear

15 GCATCGTCGACTTTGAGCTCCAGCTTGGTCCC

INFORMATION FOR SEQUENCE NO 7:

20 Sequence characteristics
length : 87 bases
type : nucleic acid
25 strandedness : single
topology : linear

30 CGATTAGTAAGGAGGTTTCATATGTCGACAAATCCTCAGGATCTGGCTCC
GAATCCAAAAGCACGCAGGTCAAACCTCGAGAAGCTTG

35 INFORMATION FOR SEQUENCE NO 8:

40 Sequence characteristics
length : 87 bases
type : nucleic acid
strandedness : single
45 topology : linear

50 GATCCAAGCTTCTCGAGTTTGACCTGCGTGCTTTTGGATTTCGGAGCCAG
ATCCTGAGGATTTGTCGACATATGAAACCTCCTTACTAAT

Claims

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1. A polypeptide which possesses activity to inhibit binding of human IL-6 to human IL-6 receptor.

2. A polypeptide according to Claim 1 comprising the H-chain variable region and the L-chain variable region of an anti-IL-6 antibody.
3. A polypeptide according to Claim 2, which is obtained by joining part or all of the H-chain variable region and part or all of the L-chain variable region of an anti-IL-6 antibody using a linker.
4. A polypeptide according to Claim 3, wherein the linker is a peptide.
5. A polypeptide according to Claim 4, which is selected from the group consisting of:
 (a) a polypeptide having the following amino acid sequence:

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Met Asp Ile Leu Leu Thr Gln Ser Pro Lys Phe Leu Leu Val Ser Ala
 1           5           10           15
Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Ser Val Ser Thr
15           20           25           30
Asp Val Ser Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu
35           40           45
Ile Tyr Tyr Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr
20           50           55           60
Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Thr Val Gln
65           70           75           80
Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln Asp Tyr Arg Ser Pro
85           90           95
Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys Val Asp Lys Ser
25           100          105          110
Ser Gly Ser Gly Ser Glu Ser Lys Ser Thr Gln Val Lys Leu Glu Glu
115          120          125
Ser Gly Pro Gly Ile Leu Gln Pro Ser Gln Thr Leu Ser Leu Thr Cys
30           130          135          140
Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser Gly Met Gly Val Ser Trp
145          150          155          160

Ile Arg Gln Pro Ser Gly Lys Gly Leu Glu Trp Leu Ala His Ile Tyr
165          170          175
Trp Asp Asp Asp Lys His Tyr Asn Pro Ser Leu Lys Ser Arg Leu Thr
180          185          190
Ile Ser Lys Asp Thr Ser Thr Asn Gln Val Phe Leu Lys Ile Thr Ser
195          200          205
Val Asp Thr Ala Asp Thr Ala Thr Tyr Phe Cys Ala Arg Arg Ser Leu
210          215          220
Tyr Gly Asn Trp Gly Asp Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
45           225          230          235          240
Ser Val Thr Val Ser Ser
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- (b) a polypeptide which in respect to (a) is deficient in one or more amino acids;
- (c) a polypeptide in which in respect to (a) one or more amino acids are replaced;
- (d) a fusion peptide comprising a polypeptide according to (a), (b) or (c) in which the additively connected amino acids do not interfere with the inhibition activity of the polypeptide or may be easily eliminated;
- (e) a polypeptide in which in respect to (a) one or more amino acids are chemically modified.

6. A polypeptide according to Claim 5, which lacks the N-terminal Met.

7. A polypeptide according to Claim 5, in which the chemical modification comprises acetylating, amidating or adding of polyethylene glycol.
8. A DNA-sequence coding for a polypeptide according to any of the Claims 1 to 6.
9. A DNA-sequence according to Claim 8 which is selected from the group consisting of:
 (a) a DNA-sequence having the following nucleotide sequence;

ATG GAC ATC CTG CTG ACA CAG TCT CCA AAA TTC CTG CTT GTA TCA GCA 48
 GGA GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT ACT 96
 GAT GTA AGT TGG TAC CAA CAG AAG CCA GGG CAG TCT CCT AAA CTA CTG 144
 ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC TTC ACT 192

GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG 240
 GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGG TCT CCA 288
 TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA GTC GAC AAA TCC 336
 TCA GGA TCT GGC TCC GAA TCC AAA AGC CAG GTC AAA CTC GAG GAG 384
 TCT GGC CCT GGG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT TGT 432
 TCT TTC TCT GGG TTT TCA CTG AGC ACT TCT GGT ATG GGT GTG AGC TGG 480
 ATT CGT CAG CCT TCA GGG AAG GGT CTG GAG TGG CTG GCA CAC ATT TAT 528
 TGG GAT GAT GAC AAA CAC TAT AAC CCA TCC CTG AAG AGC CGG CTC ACA 576
 ATC TCC AAG GAT ACC TCC ACC AAC CAG GTA TTC CTC AAG ATC ACC AGT 624
 GTG GAC ACT GCA GAT ACT GCC ACA TAC TTC TGT GCT CGA AGA AGT CTC 672
 TAT GGT AAT TGG GGG GAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC 720
 TCA GTC ACC GTC TCC TCA 738

- (b) a DNA-sequence which in respect to (a) is deficient in one or more nucleotides;
 (c) a DNA-sequence in which in respect to (a) one or more nucleotides are replaced;
 (d) a DNA-sequence to which in respect to (a) one or more nucleotides are added.
10. A vector comprising a DNA-sequence according to Claim 8 or 9.
11. A vector according to Claim 10, which is a plasmid.
12. A plasmid which is pFv(HH61-10).
13. A cell transformed with a DNA-sequence according to any of the Claims 8 to 9.
14. A cell transformed with a vector according to any of the Claims 10 to 12.
15. A cell according to Claim 13 or 14 which is an eucaryote.
16. A cell according to Claim 13 or 14, which is a procaryote.
17. A cell according to Claim 16 which is Escherichia coli.
18. E.coli FERM-BP 4523.
19. A method for producing a polypeptide according to any of the Claims 1 to 7, characterized by culturing a transformant according to any of the Claims 13 to 17 to produce said polypeptide, and collecting said polypeptide.
20. An IL-6 suppressant comprising a polypeptide according to any of the Claims 1 to 7.

21. An agent for the treatment of autoimmune diseases comprising a polypeptide according to any of the Claims 1 to 7.

22. An anti-inflammatory agent comprising a polypeptide according to any of the Claims 1 to 7.

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23. An anti-cancer agent comprising a polypeptide according to any of the Claims 1 to 7.

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(Name of Document) DRAWINGS

Fig. 1

(a) 5'-CAGGTGAAACTCGAGCAGTCAGG-3'

CC G G T

(b) 5'-AAGCTTCATGAGGAGACGGTGACCGTGGTCCC-3'

(c) 5'-ACAGTCATAATGTCCCATATGGACATTCTGCTGACACAGTCTCCA-3'

T C A A C

(d) 5'-GCATCGTCGACTTTGAGCTCCAGCTTGGTCCC-3'

(a) shows the 5' end primer of the H-chain.

(b) shows the 3' end primer of the H-chain.

(c) shows the 5' end primer of the L-chain.

(d) shows the 3' end primer of the L-chain.

(a) and (c) primers are the mixture of 32 primers that are degenerate at five positions. Underlined sequences of (b) primer represent a termination codon.

Fig. 2

<Clal>

<NdeI><SalI>

5'-CGATTAGTAAGGAGGTTTCATATGTCGACAAATCCTCAGGATCTGGCTCCGAATCCAAA

3'-TAATCATTCTCTCAAAGTATACAGCTGTTTAGGAGTCCTAGACCGAGGCTTAGGTTTT

<XhoI><HindIII><BamHI>

GCACGCAGGTCAAACCTCGAGAAGCTTG -3'

CGTGGTCCAGTTTGAGCTCTTCGAACCTAG-5'

Fig. 3

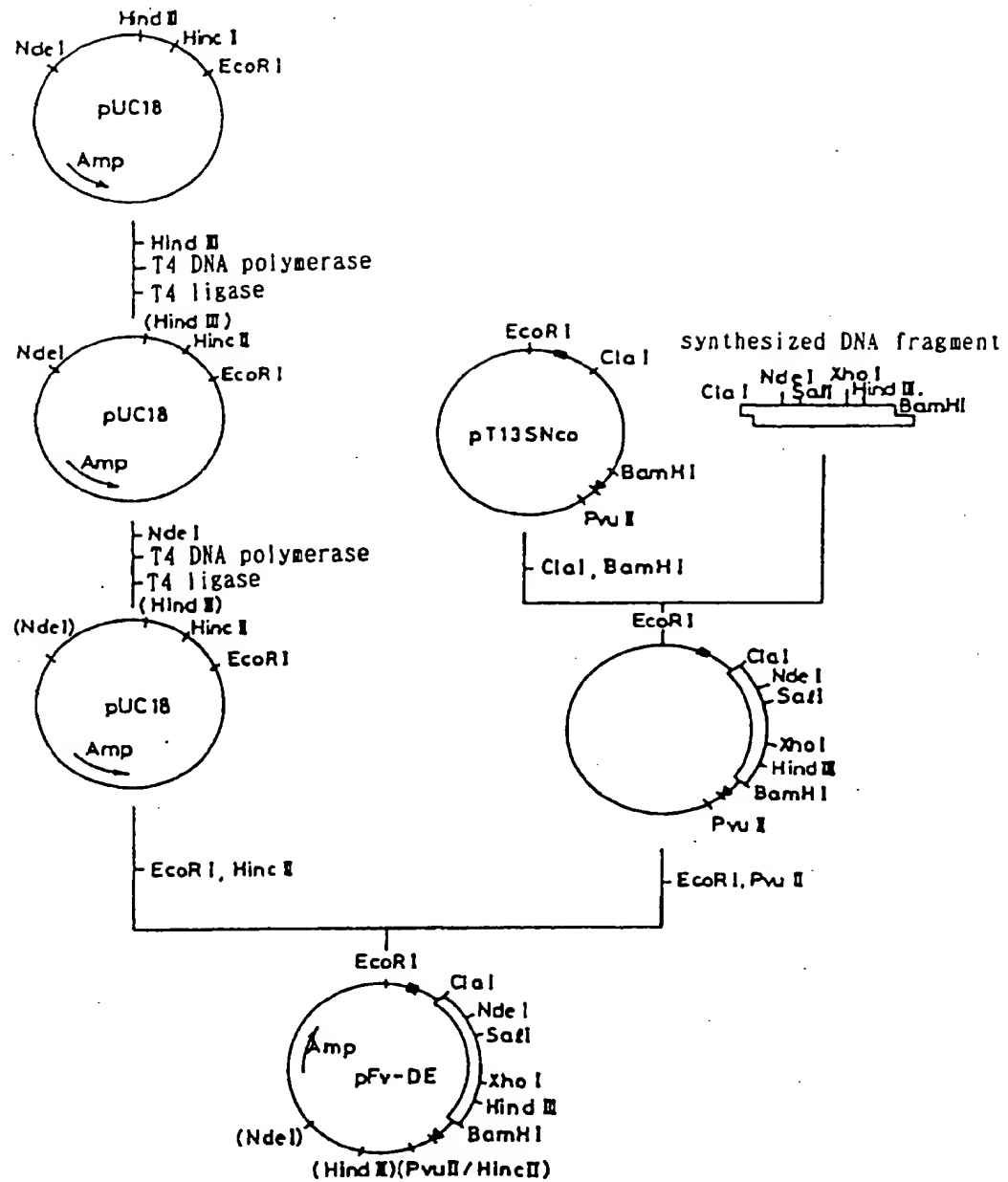
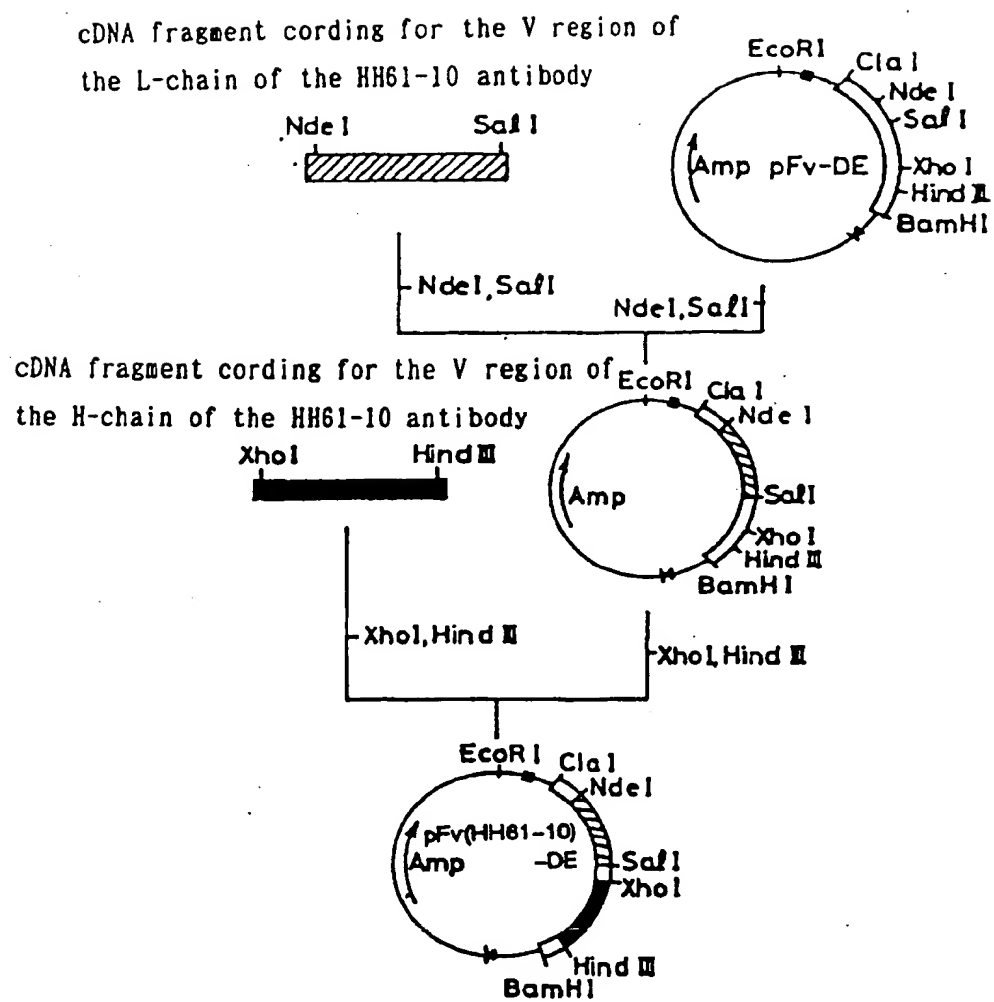


Fig. 4



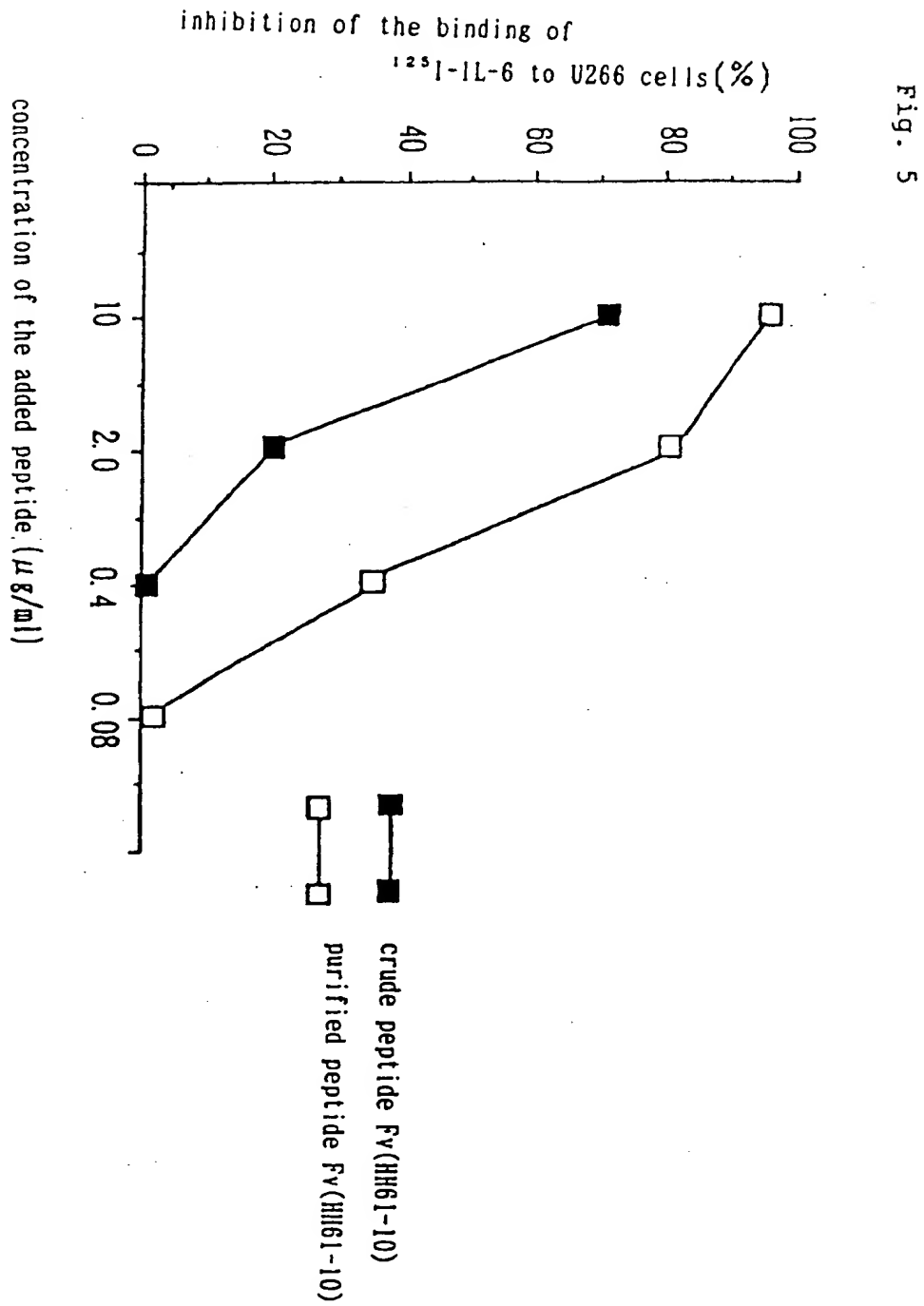


Fig. 6

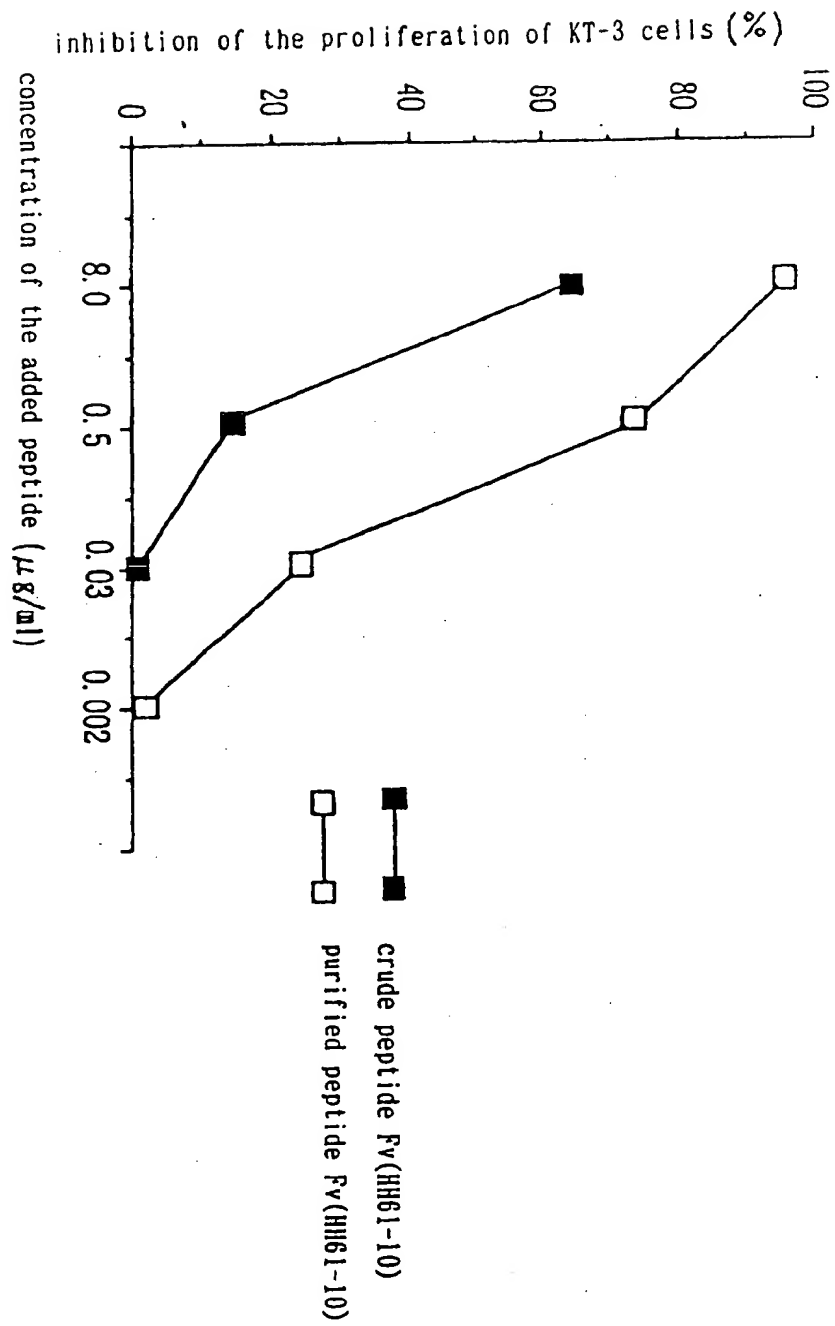
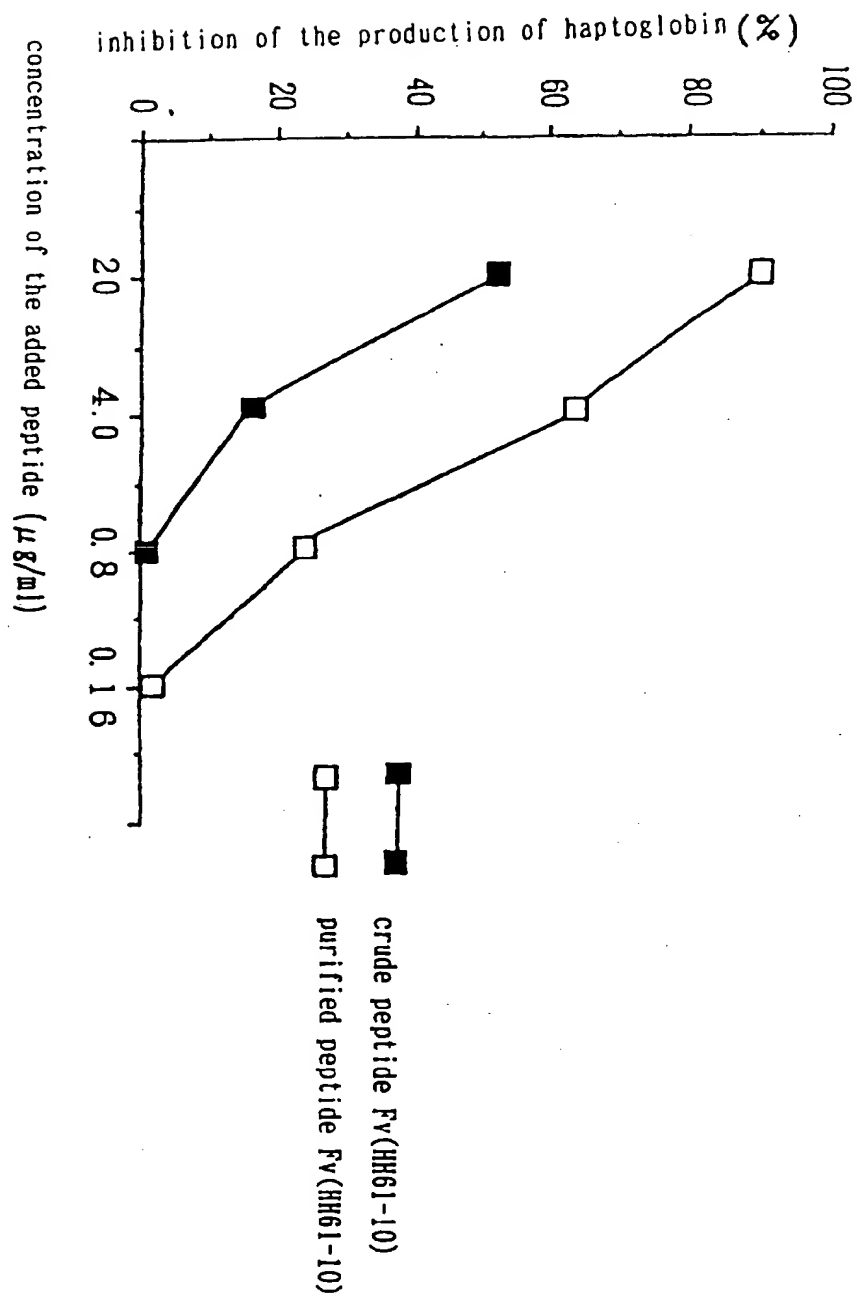


Fig. 7



(19)



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C07K 13/00, C12P 21/08,
C12N 5/10, C12N 1/21,
A61K 39/395**

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(71) Applicant: **Ajinomoto Co., Inc.**
No. 15-1, Kyobashi 1-chome
Chuo-ku
Tokyo (JP)

(72) Inventor: **Shimamura, Toshiro, c/o Central**
Research Lab.
Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku,
Kawasaki-shi,
Kanagawa-ken (JP)

Inventor: **Nakazawa, Harumi, c/o Central**
Research Lab.

Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku,
Kawasaki-shi,
Kanagawa-ken (JP)

Inventor: **Hamuro, Junji, c/o Central Research**
Lab.

Ajinomoto Co., Inc.,
No. 1-1 Suzuki-cho
Kawasaki-ku,
Kawasaki-shi,
Kanagawa-ken (JP)

(74) Representative: **Strehl Schübel-Hopf Groening**
& Partner
Maximilianstrasse 54
D-80538 München (DE)

(54) **Polypeptide capable of inhibiting the binding between human IL-6 and its receptor.**

(57) A peptide which inhibits the binding of human IL-6 to human IL-6 receptor, a human IL-6 suppressant, anti-inflammatory agent, immunosuppressant or treatment for autoimmune diseases containing the peptide, a gene which codes for the peptide, a plasmid carrying the gene, a transformant carrying

the plasmid, and a method for the production of the above peptide by culturing the above transformant.

The peptide is useful as a treatment for diseases thought to be induced or aggravated by IL-6.

EP 0 617 126 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 10 2346

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X	WO-A-91 07986 (SCHERING CORP ;FLETCHER STARNES H (US)) * page 3, line 31 - page 4, line 7 * * claims * ---	1-4, 20-23	C12N15/13 C07K15/28 C07K13/00 C12P21/08 C12N5/10 C12N1/21 A61K39/395
X	WO-A-91 08774 (CETUS CORP ;AARDEN LUCIEN A (NL)) * page 10, line 19 - line 29 * * claims 1,2,4,8-10 * * examples * ---	1-4, 20-23	
X	EP-A-0 410 813 (FUJIREBIO INC.;KISHIMOTO, TADAMITSU) * page 3, line 57 - page 4, line 12 * * examples * * claims * ---	1,2, 20-23	
X	DE-A-39 39 706 (CENTRE REGIONAL DE TRANSFUSION SANGUINE;BIOTEST PHARMA GMBH) * examples * * claims * ---	1,2, 20-23	
X	MOLECULAR IMMUNOLOGY, vol. 28,no. 11, November 1991 OXFORD, GB, pages 1155-1161, T. SHIMAMURA ET AL. 'Analysis of interleukin 6 (IL-6)/IL-6 receptor system using monoclonal anti-IL-6 antibodies.' * the whole document * ---	1,2, 20-23	TECHNICAL FIELDS SEARCHED (Int.Cl.5) C12N C07K C12P A61K
A	NATURE, vol. 347, 4 October 1990 LONDON, GB, pages 497-498, A. PLÜCKTHUN 'Antibodies from Escherichia coli.' * page 497, right column, line 40 - line 58 * --- -/--	3-19	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 7 April 1995	Examiner Nooij, F
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 10 2346

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 25, 5 September 1991 BALTIMORE MD, USA, pages 16343-16349, Y. LAROCHE ET AL. 'Characterization of a recombinant single-chain molecule comprising the variable domains of a monoclonal antibody specific for human fibrin fragment D-dimer.' * abstract *	3-19	
			TECHNICAL FIELDS SEARCHED (Int.Cl.5)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 7 April 1995	Examiner Nooij, F
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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